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SUCRASES IN FUNGI[1]

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Abstract

The yields of the extracellular sucrases produced by fungi and yeasts were markedly increased by using sucrose monopalmitate as carbon source. The sucrases of 10 fungi moved as single components in zone electrophoresis. The sucrases of six fungi were composed of two separable components. In one fungus, three sucrase components were observed.

Introduction

Recently we have shown (1) that cellobiose is the natural inducer of cellulase in fungi, and that it acts best as an inducer under conditions where it is supplied slowly, as in cellulose cultures. Another means of supplying cellobiose slowly is by using insoluble cellobiose octaacetate as a substrate. Many fungi produce an esterase hydrolyzing the acetate groups from this, slowly liberating cellobiose. These fungi give much higher cellulase yields when grown on cellobiose octaacetate than when grown on cellobiose. This observation led us to speculate that sucrose monopalmitate might act in a similar way as an inducer of sucrase. We have tested and confirmed this hypothesis with a number of organisms from the Quartermaster Culture Collection. Some observations on the properties of the fungal sucrases are also being reported.

Methods

Fungi were grown at 29° C in shake culture on a standard nutrient salts medium (2), plus a carbon source. Sucrase and other enzymatic activities were determined on the cell-free culture filtrates. One unit of enzyme activity is that quantity which, in 2 ml of assay solution, produces 1.0 mg of reducing sugar measured with the dinitrosalicylic acid reagent (DNS, see reference 3) as glucose, in 1 hour at 50°. Assays were at pH 4.8 in phosphate (or citrate) buffer. Substrates were used at 2.0 mg per ml of assay solution for disaccharides and polysaccharides and 3 mg per ml for trisaccharides. Michaelis constants (K_m) were estimated from plots of the reciprocal of the velocity against the reciprocal of the substrate concentration (4). Initial rates of reactions were determined by incubation for 15 to 30 minutes.

Transfer reactions were followed by incubating enzymes with sucrose (1.0–7.5%). Samples were withdrawn at various times, spotted on paper, and developed with isopropanol, acetic acid, water (27:4:9). Carbohydrates were detected with benzidine (5) plus trichloroacetic acid, and fructose-containing sugars with resorcinol–HCl (6).

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The enzyme samples used for zone electrophoresis (7, 8) were obtained as acetone precipitates of the culture filtrate. These were dissolved and dialyzed against water, evaporated to dryness *in vacuo* at 30° C, and taken up in phosphate buffer of pH 7.0 and ionic strength 0.1. After electrophoresis at 8 v per cm for 20 hours, the 100-cm starch block was cut into 100 sections of equal length. Each section was eluted with 6 ml water containing 0.01% bovine plasma albumin (9). The eluates were assayed for enzymic activity. The time of assay varied with the amount of enzyme present. Quantitative variations among the different activities were handled by varying the time of incubation (1–1200 minutes) and the wavelength at which the DNS solutions were read in the colorimeter. For this reason, the heights of the activity peaks of enzymes acting on different substrates are not directly comparable. The primary purpose of the curves is a determination of the number of components present in each preparation, rather than a strictly quantitative comparison of the various enzymes.

Results

(A) Screening of Fungi for Sucrase Production

Preliminary tests showed that sucrase yields were much higher when organisms were grown on sucrose monopalmitate than when they were grown on sucrose or on other carbon sources. As a result, 140 fungi were screened on a medium containing 0.4% sucrose monopalmitate and 0.1% sucrose. Many of these secreted over 30 units/ml into the medium (Table I). No measure of intracellular enzyme was made. Under the test conditions many fungi produced more sucrase than *Saccharomyces cerevisiae*, the usual source of invertase. Particularly good yields were obtained from some *Penicillium* spp. and from *Candida utilis*. The yields shown here are not maximum values. At the termination of the experiments (21 days), the enzyme concentrations were continuing to rise, some species producing as much as 1000–3000 units/ml (*Penicillium brefeldianum*, *P. parvum*, *P. quadrilineatum*, *P. melinii*, and *C. utilis*). These yields indicate that crude filtrates can be obtained which have 1/10th the activity of the concentrates distributed as commercial invertase (Nutritional Biochemical Co.).

Seven of the more productive fungi were compared for sucrase production on a variety of substrates (Table II). The best yields were obtained on sucrose monopalmitate. The maximum effect was observed with *Aspergillus wentii*. It was anticipated that sucrose octaacetate would give results similar to those for the monopalmitate, but this was not so. Glucose pentaacetate, included mainly as a check on sucrose octaacetate, was surprisingly quite a good stimulant of sucrase production for three of the fungi. Glycerol and mannose had a favorable effect on one organism, but on no other substrate were the yields as high as those obtained on the monopalmitate. Palmitic acid plus sucrose could not be substituted for sucrose palmitate (data not shown), the yields for this combination being of the magnitude of those obtained on sucrose.

TABLE I

Sucrase yields of fungi grown on sucrose palmitate (0.4%) + sucrose (0.1%)

Organism	Enzyme, units/ml				Ratio, S/R	$K_{m\ (sucrose)}$, *M*
	QM	Sucrase (S)	Raffinase (R)	Melezitase		
Alternaria tenuis	7158	30	1	0.2	30	
Aspergillus awamori var. *hominis*	6949	50	11	1.5	5	
A. flavus	6737	120	52	0	2	0.011
A. niger	877	144	28	2.5	5	0.035
A. oryzae	6735	66	30	0	2	0.0048
A. quadricinctus	6874	104	54	0.4	2	
A. wentii	6729	88	15	0.5	6	0.017
Candida utilis	B1487	120	8	0	15	
C. utilis	B1505	250	16	0.1	16	0.009
Circinella umbellata	1965	32	12	0	3	0.014
Cunninghamella echinulata	6782	208	12	0	17	0.009
Fusarium sambucinum	7162	64	30	0	2	0.0026
Penicillium brefeldianum	1873	1600	54	0.4	30	0.0005
P. melinii	1931	104	18	0	6	0.0064
P. nigricans	1933	67	4	0.1	15	
P. ochro-chloron	477	325	35	1.5	9	0.0006
P. parvum	1878	220	11	0	20	0.0006
P. quadrilineatum	7871	1080	240	0.2	5	0.0046
P. queenslandicum	7870	40	1	0.3	40	0.001
P. verruculosum	3698	192	40	0	5	
Pestalotia copernica	4567	42	13	1.0	3	
Pestalotiopsis westerdijkii	381	130	14	1.0	9	0.002
Rhodotorula mucilaginosa	B1526	60	18	0.2	3	
Saccharomyces carlsbergensis Y379 (NRRL)		36	2	0.2	16	
S. cerevisiae (Carling)		75	9	0	9	0.022
Invertase (Nutr. Bioch. Co.)		32000	—	—	—	0.024

TABLE II

Effect of various carbon sources on sucrase production

Organism	Sucrose palmitate*	Yield in units/ml, when grown on: Sucrose octaacetate*	Glucose pentaacetate*	Glycerol*	Mannose*	Sucrose†
Aspergillus niger	877	6	5	3	3	7
A. wentii	44*a*	1	0	1	0	NT
Candida utilis	B1505	16	18	108	66	NT
Cunninghamella echinulata	6782	22	120	16	1	NT
Penicillium melinii	1931	10	11	3	3	28
P. parvum	1878	66	42	19	19	6
Pestaloiopsis westerdijkii	381	13	52	4	12	4

NOTE: NT = No test.
*Substrate 0.4%, plus sucrose 0.1%.
†Sucrose alone 0.5% (separate experiment). Time 21 days; shaken.

Dry preparations of sucrase, prepared by acetone precipitation from the culture solution, varied in activity from 16 to 384 sucrase units/mg. These preparations were used to prepare the "sucrase solutions" employed throughout the remainder of this work. The recovery of activity was over 60% in about half of the tests. In two instances it was above 150%. Such apparent activation has been observed previously with some of our cellulase and β-1,3-glucanase solutions, particularly in cultures which had been incubated beyond the time of maximum activity. The poorest recovery of activity in an acetone precipitate was 14%.

(B) Nature of Sucrose Monopalmitate

Commercial sucrose monopalmitate (Colonial Sugars Co.) is reported as a monoester substituted predominantly at the 6-position of the glucose portion of the molecule. When subjected to the action of our sucrase solutions, it is hydrolyzed very slowly, about 1/1000 as fast as sucrose. It is probable that esterases are limiting, and that in the absence of these, sucrose palmitate is resistant to the sucrases. Sucrose palmitate had no inhibiting effect on the hydrolysis of sucrose by sucrases (data not shown).

The extent of hydrolysis of sucrose monopalmitate by our enzyme solutions, as measured by reducing sugar production, was 49%. These data suggest that only part of the ester groups can be hydrolyzed by the esterases occurring in our sucrase solutions. The remaining substituents must be so situated as to resist enzymatic action, even though they are susceptible to hydrolysis by acid. This indicates that an appreciable portion of the substitution is not at the 6-position as reported above.

(C) Properties of Fungal Sucrases

The sucrose concentration required for obtaining the maximum rate of hydrolysis varies with the source of enzyme. For sucrases of some *Penicillium* spp., such as *P. brefeldianum*, this value is as low as 2 to 3 mg sucrose/ml. When the concentration greatly exceeds this, marked inhibition is observed (Fig. 1). For other sucrases, such as that of *C. utilis*, the rate of hydrolysis increased with substrate concentration over the range tested. K_m values, estimated for most of these sucrases, are comparable to those reported by others for sucrases of the same species. For example, our value for sucrase of the yeast *Saccharomyces cerevisiae* is 0.022 *M*; that found by Michaelis and Menten is 0.016 *M* (10); and that by Myrbäck, 0.04 *M* (11). However, the values vary from one organism to another, from 0.0005 *M* in *P. brefeldianum* to 0.035 *M* in *A. niger* (Table I).

The sucrases of 10 fungi and yeasts tested have an optimum activity in phosphate buffer at about pH 5. Activity at pH 2.0 and at pH 8.0 are usually quite low. An exception is found in *A. niger* where activity at pH 2.0 is about 50% of that at the optimal pH. Activities in citrate and in phthalate are similar to those in phosphate. Activity in acetate is somewhat less.

The effect of temperature on the sucrase activities of six fungi was tested over the range 20–60° C. Four of these (*A. niger*, *Pestalotiopsis westerdijkii*, *C. utilis*,

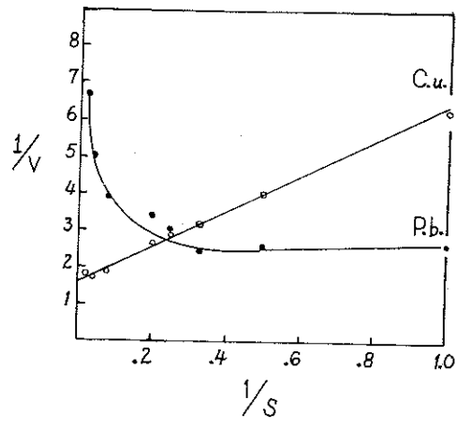

FIG. 1. Effect of sucrose concentration on rate of hydrolysis. ○ *Candida utilis* QM B1505 sucrase; ● *Penicillium brefeldianum* QM 1873 sucrase. Conditions: *M*/20 PO_4; 35°. S = mg sucrose/ml; V = mg reducing sugar/ml/15 minutes.

and *P. brefeldianum*) were most active at 60° C during the 30-minute incubation period. The time:activity curves showed no deviation from a straight line even at 60° C. The other two preparations (*P. melinii*, *Cunninghamella echinulata*) were most active at 50° C, inactivation at 60° C becoming apparent within 10 minutes.

During the course of these experiments some rather unusual results were observed. The source of difficulty was found to be the presence of merthiolate in the buffer. Over 50% inhibition of many of the sucrases occurred at a concentration of 0.01% of this organic mercury compound. Except for β-amylase, merthiolate had not affected the activity of glucanases studied previously. Cysteine overcomes the inhibitory effect.

(D) Electrophoretic Separation of Sucrases

The sucrase in most of the 17 enzyme preparations subjected to zone electrophoresis moved as a single component. There were seven examples of preparations having at least two active components (Table III). Three of those illustrated (Figs. 2 and 3) exhibited clear-cut separations. All peaks shown (Figs. 2 and 3) represent peaks of enzyme activity, and not of protein. They give no information on specific activity (units/mg protein). In order to define the major peaks clearly, it is necessary to limit the hydrolysis assay time. This may obscure minor components. Thus the *P. melinii* sucrase at 8 cm appears to have no raffinase activity (Fig. 2). Increasing the hydrolysis time by a factor of 10 did reveal activity. The *Alternaria tenuis* patterns show two sucrases (Table III) differing markedly in sucrase/raffinase ratios.

The sucrase preparations contained many enzymes, some known, some unknown. When the original preparation was found to contain maltase, β-2,1-fructanase, β-2,6-fructanase, or other enzymes having a bearing on the sucrase

TABLE III

Sucrase components from electrophoresis

Source	QM	Components No.	(cm)†	Enzyme activities (units/ml) on:* Su	Ra	Fructan β-2,1	Fructan β-2,6	Ma	Me
Alternaria tenuis	7158	2	23	2.6	0.02	0	0	0	0
Alternaria tenuis	7158		15	0.5	0.1	0	0	0	0
Aspergillus niger	877	1	8	13.1	4.8	0.4	0.1	0	0
A. sydowi	31c	1	35	3.0	0.2	0	0	0	0
A. wentii	44a	1	11	60.0	50.0	0.1	0.1	0	0
				82.0	6.3	0	0.2	0	0
Candida utilis	B1487	1	10	32.8	4.4	0.1	0.2	0	0
C. utilis	B1505	1	13	13.0	3.9	0	0	0	0
Cunninghamella echinulata	6782	1	22						
				92.0	6.7	0.2	0.1	0	0
Penicillium brefeldianum	1873	1	1	7.0	0.2	0	0	0	0
P. melinii	1931	2	16	6.0	2.4	0.8	0.4	0	0
P. melinii	1931		4	6.6	0.9	0.4	0.4	0	0
P. nigricans	1933	2	11	8.5	0.7	0.1	0.1	0	0
P. nigricans	1933		8	19.0	1.2	0.5	0.2	0.1	0.1
P. ochro-chloron	477	2	6	16.0	0.6	0.1	0	0.1	0
P. ochro-chloron	477		4	5.2	0.3	0	0	0	0
P. parvum	1878	2	6	10.5	0.5	NT	NT	0	0
P. parvum	1878		2	32.0	13.0	1.2	0.8	0	0
P. quadrilineatum	7871	3	7	14.0	3.2	0.5	0.3	0	0
P. quadrilineatum	7871		9	11.0	1.6	0.2	0.3	0	0
P. queenslandicum	7870	1	7	1.1	0	0	0	0	0
P. verruculosum	3698	1	14	21.0	3.5	2.1	1.6	0.2	0
Pestalotiopsis westerdijkii	381	2	20	1.1	0.6	0.1	0	0	0
Pestalotiopsis westerdijkii	381		8	NT	NT	NT	NT	NT	NT
Saccharomyces cerevisiae (Invertase, Nutr. Bioch. Co.)		1	8	80.0	10.0	0.1	0.4	0	0

*Sucrose (Su), raffinose (Ra), maltose (Ma), melezitose (Me).
†All components moved towards the anode.

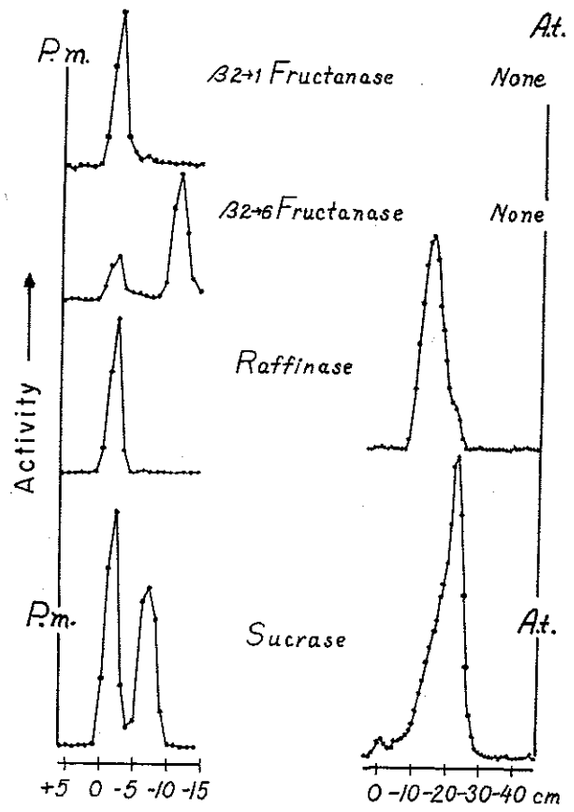

FIG. 2. Electrophoretic patterns of sucrases. P.m. = *Penicillium melinii* QM 1931; A.t. = *Alternaria tenuis* QM 7158. Length of incubation (minutes)—*P. melinii* fractions: sucrase 5, raffinase 10, β-2,6-fructanase 40, β-2,1-fructanase 45; *A. tenuis* fractions: sucrase 25, raffinase 180, β-2,6-fructanase 1080, β-2,1-fructanase 1080.
Abscissa shows distance of component from starting point in centimeters.

problem, the electrophoretic fractions were analyzed for these. Maltase was of particular interest. In the *A. wentii* (Fig. 3) preparation, maltase moved at a rate distinctly different from that of sucrase. In the *A. niger* preparation, the maltase was composed of three components, the sucrase of only one. Amylase was present in several preparations. In that of *A. sydowi*, six distinct amylase components were found, the fastest of these moving 40–50 cm in 20 hours.

(E) Specificity of Electrophoretically Separated Sucrases

All of the sucrase components tested transfer β-fructosyl groups to water, and usually to sucrose. All act on raffinose (R) as well as on sucrose (S), but the ratio of activities on these two substrates (S/R) varies from one organism to another. In *P. melinii*, where the two sucrases have been best separated

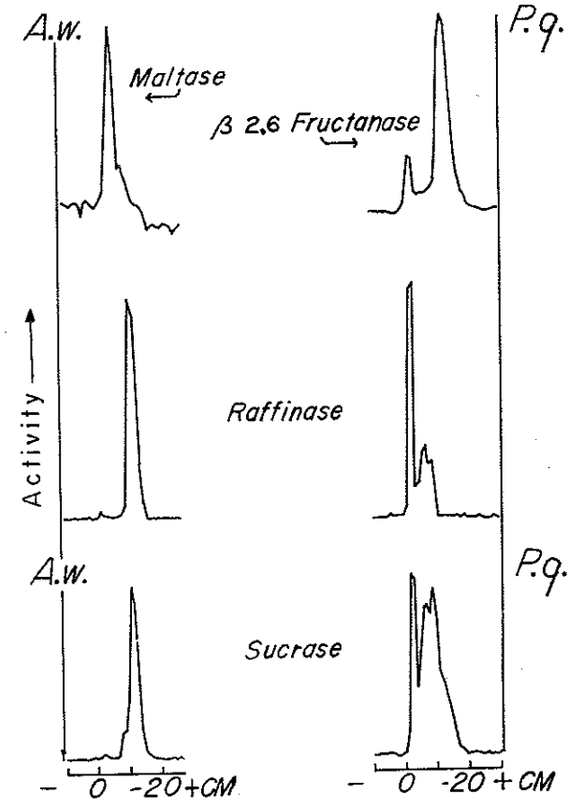

FIG. 3. Electrophoretic separation of sucrases. A. w. = *Aspergillus wentii* QM 44*a*; P. q. = *Penicillium quadrilineatum* QM 7871. Length of incubation (minutes)—*A. wentii* fractions: sucrase 1, raffinase 3, maltase 180; *P. quadrilineatum* fractions: sucrase 2, raffinase 2, β-2,6-fructanase 30.
Ordinate = enzymatic activity; abscissa = distance of enzyme from starting point; + = anode end of block; − = cathode end.

electrophoretically, the S/R ratios differ by a factor greater than 10. The lowest S/R value found (= 1.0) is for *A. wentii* sucrase; the highest, over 50, was found in electrophoretic fractions of *A. tenuis* and of *Penicillium queenslandicum* (Table III). Fifteen of the electrophoretically purified sucrases (including commercial invertase) were allowed to act on 1.0% raffinose for 20 hours and the hydrolyzates subjected to paper chromatography. In all cases, the major products were melibiose and fructose, indicating β-fructosidase activity.

Fructosyl transfer to sucrose varies for sucrases of different fungi or even for the two sucrases of a single fungus (*P. melinii*). Usually, a trimer can be detected, but in at least three instances, checked several times, no trimer accompanied the hydrolysis in (1) *P. melinii* slow component, (2) *A. niger*, (3) *C. echinulata*. On the other hand, under the same conditions, a few organ-

isms produced tetramer as well as trimer (judged by movement on paper chromatograms only): (1) *P. melinii* fast component, (2) *P. brefeldianum*, (3) *P. queenslandicum*.

The sucrases often had activity on either or both of the fructans, but the rate of activity on the long chains is usually very low compared to that on sucrose (Figs. 2 and 3). Some sucrases appear to have no activity on fructans, and some fructanases little or no activity on sucrose. Again, increasing the sensitivity of the assays by lengthening the incubation time might reveal traces of these activities.

No electrophoretically homogenous sucrase had any appreciable activity on maltose or on melezitose (Table III).

Discussion

Sucrase, for many years, was considered to be an intracellular enzyme. Dworschack and Wickerham (12) now have shown that the proportion of invertase in the organism to that in the medium varies from one yeast to another and from one environment to another. Of the yeasts examined, these authors found *C. utilis* to be one of the best producers of extracellular sucrase. Our tests confirm this, ranking this yeast with the fungi *Penicillium brefeldianum*, *P. parvum*, *P. quadrilineatum*, and *P. melinii*. On the other hand, *P. spinulosum* and *A. oryzae*, species used by Bealing and Bacon (13), have good activity, but are not nearly as active as those listed above when grown under our conditions.

The yields of sucrase were greatly increased by growing the organisms on sucrose monopalmitate. The stimulating value of this material seems to lie in the slow liberation of sucrose through action of a fungal esterase. Early workers have claimed that small periodic additions of sucrose give better yields than one large dose (14). *Pestalotiopsis westerdijkii*, a strong producer of esterase, is least stimulated, apparently because the sucrose monopalmitate is too rapidly converted to sucrose and palmitic acid. Whereas the stimulatory effect of the monopalmitate was anticipated from earlier results on cellulase stimulation by use of cellobiose octaacetate (1), our other guesses did not turn out as well. Glucose pentaacetate is a far better inducer than sucrose octaacetate, but neither is nearly as good as the sucrose monopalmitate.

Our data support the current view that the sucrases of fungi and yeast are β-fructosyl transferases (13). While there are some sucrases that do not readily form a fructosyl-sucrose, these do act on raffinose to yield melibiose and fructose, the fructosyl transfer being to water. None of the sucrase activity can be attributed to an α-glucosidase (maltase). When the original culture solutions had α-glucosidase activity, this enzyme separated from the sucrase during zone electrophoresis.

The electrophoretic data indicate that some fungi produce a single sucrase, some produce at least two. Three, and possibly more, sucrase components appear in electrophoretic patterns of enzyme preparations of *Fusarium moniliforme*, *Penicillium funiculosum* (7), and *P. quadrilineatum*. In these, some of the sucrase activity may be attributable to the β-fructanases present.

When more than a single sucrase component is found in an electrophoretogram, it is desirable to find some difference in these enzyme preparations other than rate of migration. Usually the ratio of activity on sucrose to that on raffinose is different for the separate components. In *P. melinii* the ability to produce a fructosyl-sucrose is much greater for one component than for another. Other differences no doubt exist.

The sucrases isolated electrophoretically have raffinase activity, but the ratios of the sucrase to raffinase activity vary widely. These enzymes may also act on β-2,1-fructan and(or) β-2,6-fructan, but always at rates far less than those on sucrose or raffinose. The electrophoretic patterns often show, in addition, a separate β-2,6-fructanase, usually with little or no effect on sucrose.

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